# Apoptosis and Remodeling of Mammary Gland Tissue during Involution Proceeds through p534ndependent Pathways'

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## Abstract

**Mammary gland involution is a physiological process that follows lactation and results in the rapid disappearance of the entire lobule-alveolar compartment. Coincident with the onset of involution, milk protein gene expression ceases and alveolar cells undergo programmed cell death. In mammary epithelial tissue culture cells in vitro, both p53-dependent and p53-independent apoptosis pathways have been identified. We investigated whether p53 induces apoptosis during mammary gland involution *in vivo* and participates in tissue remodeling. Toward this end, we examined the process of involution in the presence and absence of functional p53 in mouse models: wild-type, transgenic mice that express SV40 T-antigen specifically in mammary tissue during pregnancy; and mice that carry nonfunctional p53 alleles in their germ line. Mammary gland whole-mount and histological analyses revealed that involution and remodeling, with the concomitant disappearance of the lobulo-alveolar structures, proceeded normally in the absence of functional p53. In addition, the absence of functional p53 did not alter the involution related pattern of bax (death inducer) gene expression or the ratio of RNAs encoding bcl-x, (death inducer) to bcl-x, (survival inducer).**

## Introduction

With the beginning of each pregnancy, the mammary gland proceeds through a defined developmental program. This includes the proliferation of lobule-alveolar structures, their terminal differentiation prior to parturition, a lactational period, and involution, which is initiated at the end of the lactation and results in a glandular structure similar to that of a mature virgin. During involution, the mammary gland undergoes remodeling, which coincides with a disruption of the basement membrane, a collapse of the extracellular matrix, and PCD3 of the mammary alveolar cells (1). Early signs of involution can be seen within hours after the cessation of lactation. The number of cells undergoing apoptosis increases within the first few days and peaks at 3 days of involution. By day 5 of involution, the number of apoptotic cells decreases, and remodeling of the gland is completed by day 8 of involution. Milk protein gene expression is down-regulated with the onset of involution, and steady-state RNA levels decrease over the first 5 days of involution.

PCD is controlled by the interplay of death-promoting and -inhibiting proteins (2, 3). In the mammary gland, PCD is accompanied by increases in steady-state levels of RNA encoding bax (death inducer) and an increase in the bcl-x, (death inducer):bcl-x, (survival inducer) expression ratio (4).[4] The cell cycle control protein p53 can stimulate PCD and is a transcriptional activator of the bax gene (5). Expression of p53 has been reported to increase during mammary gland involution (l), and overexpression of p53 during pregnancy precipitates the abnormal appearance of apoptosis during pregnancy (6). Apoptosis in immortalized mammary epithelial cells in culture can be initiated by both p53-dependent and p53-independent mechanisms (7). In vivo data indicate that both pathways can be activated in mammary epithelial cells during pregnancy (4, 6). An unanswered question is whether p53-dependentt apoptosis is invoked only when cell damage is sustained by an oncogenic insult or if it is required for normal mammary gland involution. This prompted us to study whether p53 is required for normal mammary gland involution.

Mice that lack p53 are able to nurse their young (8), suggesting that p53 is not required for lactation. However, the role of p53 in the progression of mammary gland involution has not been examined. We approached this problem in two mouse models. In the transgenic model, an SV40 Tag transgene is expressed in mammary alveolar cells, beginning during pregnancy (9). For this study, only first pregnancy mice were used. Studies performed after multiple pregnancies would be complicated by the possibility of genetic selection during recurrent Tag expression. Tag expression and inactivation of p53 persisted in these mice through the

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[3] The abbreviations used are: PCD, programmed cell death; pRb, retinoblastoma protein; Tag, SV40 large T antigen; WAP, whey acidic protein; nt, nucleotide; RT-PCR, reverse transcriptase PCR.
[4] K. Heermeier and L. Hennighausen. Bax and bcl-x$_s$ are induced at the onset of apoptosis in involuting mammary epithelial cells, submitted for publication.

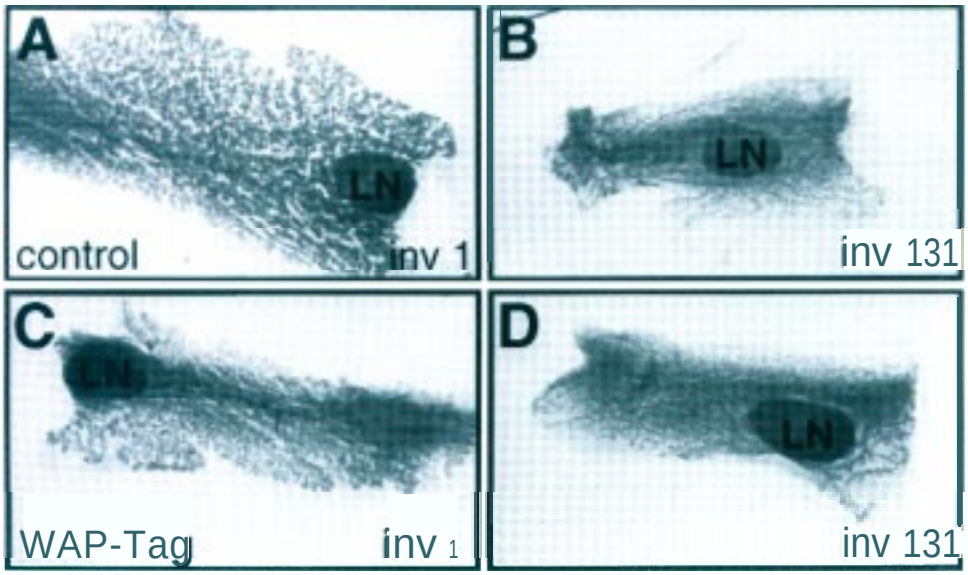



*Fig. 1.* Regression of lobulo-alveolar structures during involution in the presence of functional p53 and when the function of p53 is interrupted by Tag expression. Whole-mount analysis of mammary tissue from control *(A* and B) and Tag (C and D) mice following the first pregnancy. Inguinal glands of mice from days 1 and 13 involution were mounted on a glass slide, fixed, and stained. The entire gland is shown. LN, lymph node.

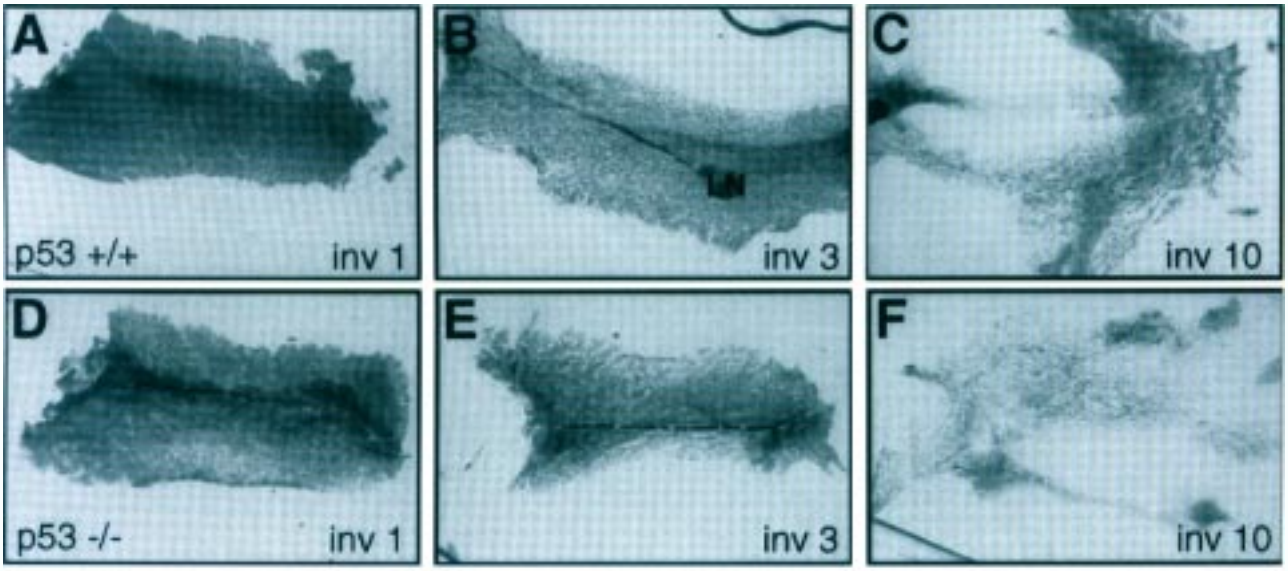
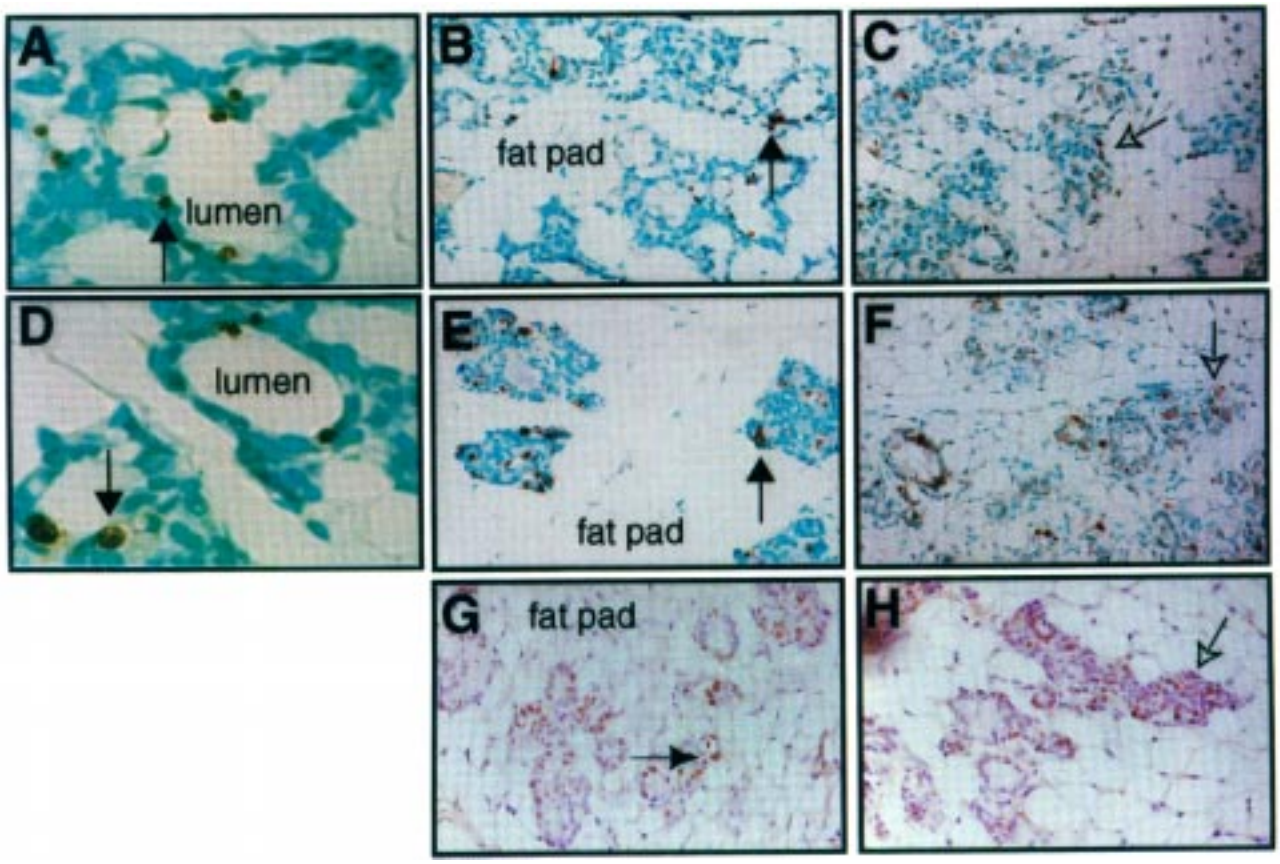



Fig. 2. Regression of lobulo-alveolar structures during involution when p53 is present and when there is a germ-line deletion of p53. Whole-mount analysis of mammary tissue from p53 +/+ *(A, B,* and C) and p53 –/– (D, E, and *F)* mice. Inguinal glands of mice from days 1 and 3 involution and abdominal glands from day 10 involution were mounted on a glass slide, fixed, and stained. The entire gland is shown. LN, lymph node.

period of involution. This model enabled us to determine whether the sudden loss of functional p53 during pregnancy in mammary epithelial cells would affect the initiation and progression of apoptosis. Importantly, the rapid inactivation of p53 in mammary cells of these mice left little opportunity for compensatory genetic modifications to occur. As a second model, we used mice that carry defective p53 alleles in their germ line (8). This model enabled us to determine whether the loss of p53 in both mammary alveolar cells and in additional cell types within the mammary fat pad interrupted the involutional process. Involution in the presence and absence of functional p53 was assessed through the examination of lobulo-alveolar and cellular structures and by monitoring the expression of genes linked to cell differentiation and PCD. Whole-mount analyses enabled us to determine whether the absence of functional p53 led to a diminished loss of cells during involution. Histological sections were used to assess whether the collapse of lobulo-alveolar structures proceeded normally over the first 3 days of involution. The appearance of apoptotic cells during involution was monitored using in situ staining. Northern blot analysis and RT-PCR were used to assess the expression of the apoptosis pathway genes, bax and bcl-x, and the milk protein genes, b-casein, *WAP,* and a-lactalbumin.




Fig, 3. Lobule-alveolar collapse and the appearance of apoptotlc cells during involution in the presence of functional p53 (control; *A-C)* and when p53 function is interrupted by Tag expression (D-H). Histological sections from mice at involution days 1 and 3 following the first pregnancy. *A-F, in situ* detection of apoptosls. G and *H,* immunohistochemlcal detection of Tag. *A,* day 1 of involution; B, day 1; *C,* day 3; D, day 1; f, day 1; *F,* day 3; G, day 1; *H,* day 3. *Solid triangular arrows, ceils* undergoing apoptosis. *Open arrows,* areas of lobulo-alveolar collapse. *So/id pinched arrow,* nuclear Tag staining. Note: the majority of nuclei in the Tag mice are positive for Tag at day 3 involution. Lumen and fat pad are indicated. Cells undergoing apoptosis appear brown.

## Results

**Mammary Gland Involution Progresses Normally in the Absence of Functional p53.** Whole-mount analyses were performed to determine if involution proceeded normally in the absence of functional p53 as measured at the level of the lobulo-alveolar structure in the whole organ. Mammary glands from Tag and control mice were examined at days 1 and 13 of involution following the first pregnancy (Fig. 1). Mammary glands from p53 −/− and p53 +/+ (control) mice were examined at days 1, 3, and 10 of involution (Fig. 2). The overall appearance of glands from the Tag and p53 −/− mice at the various days of involution was similar, if not identical, to those from control mice.

**Apoptosis of Mammary Alveolar Cells and Collapse of Lobulo-Alveolar Structures Progressed Independently of Functional p53.** Histological analyses were conducted to identify mammary alveolar cells undergoing apoptosis and to determine whether the collapse of lobulo-alveolar structures was affected by the absence of p53 (Fig. 3). In both Tag (Fig. 3, D and E) and control (Fig. 3, *A* and B) mice, numerous apoptotic cells were present at day 1 of involution. The number of apoptotic cells increased at day 3 of involution (Fig. 3, C and F). Immunohistochemical analysis demonstrated that Tag was present in the majority of the mammary alveolar cells through day 3 of involution (Fig. 3, G and H). Using specific antibodies, p53 was detected in the nuclei of mammary alveolar cells of Tag mice but not in control mice, demonstrating that p53 was sequestered by Tag (data not shown). At day 3 of involution, the lobulo-alveolar structures had collapsed in both Tag and control mice (Fig. 3, C, F, and H).

Similar histological analyses were performed in p53 -/- and p53 +/+ mice (Fig. 4). These mice had completed 3 weeks of lactation before weaning and the induction of involution. Both lobulo-alveolar collapse and increased numbers of apoptotic cells were found in p53 −/− and p53 +/+ mice after 3 days of involution (compare Fig. 4, *A, C, E,* and G to B, *D, F,* and H). One can observe fewer apoptotic cells at day 1 of involution than were found in Tag and control mice (compare Fig. 3, B and *E,* to Fig. 4, *A* and *E).* In the Tag and the control mice, involution was induced immediately after delivery by removing the pups. In contrast, involution in the p53 −/− and p53 +/+ mice was induced after 3 weeks of lactation. During these and related investigations, we have found that the rate of appearance of apoptotic cells was affected by the duration of lactation.

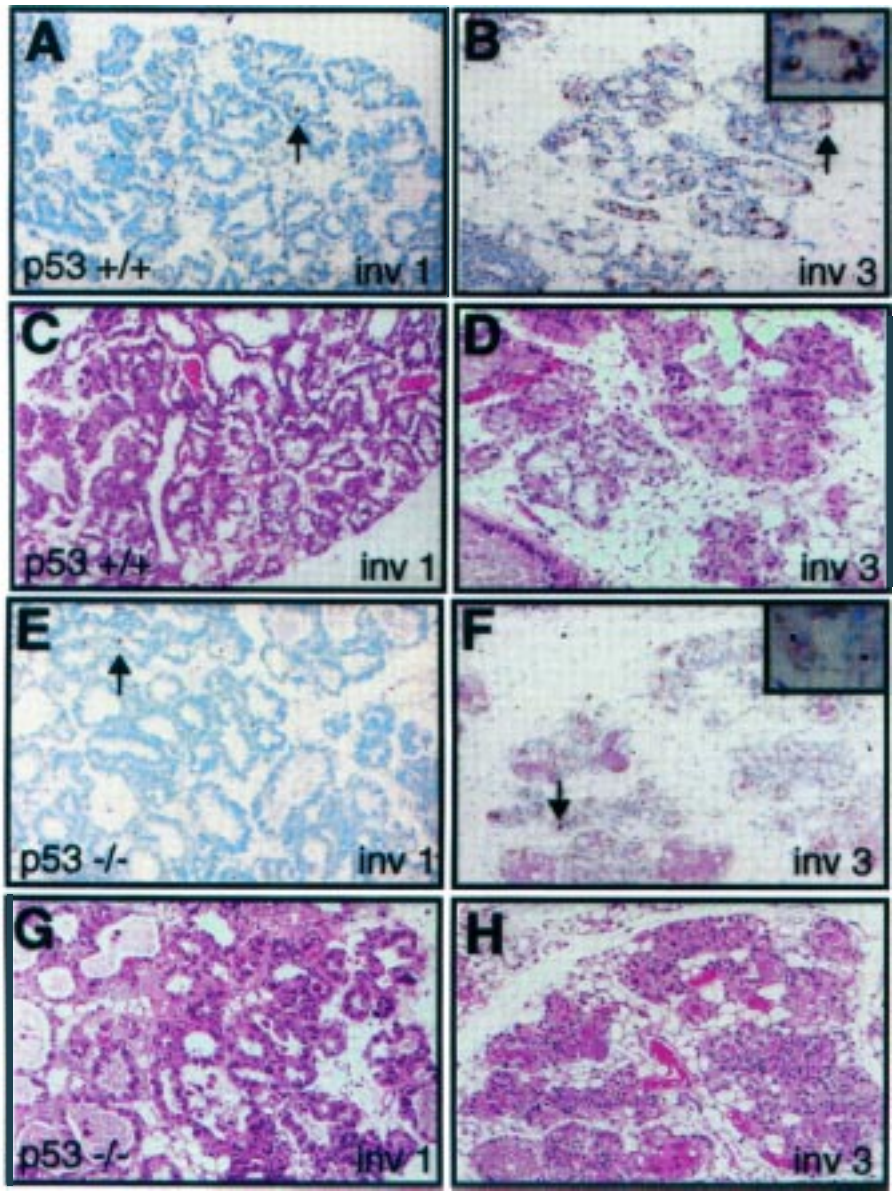
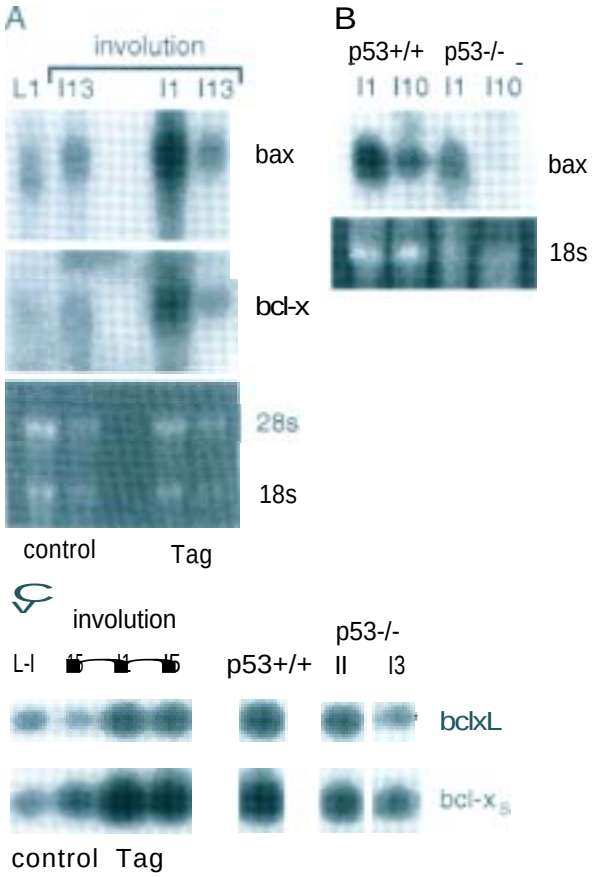



fig. 4. Lobulo-alveolar collapse and the appearance of apoptotic cells during involution when p53 is present (p53 +/+; *A-D)* and when there is a germ-line deletion of p53 (p53 –/ -; E-H). Histological sections from mice at involution days 1 and 3. A, B, E, and F, *in situ* detection of apoptosis. C, *D, G,* and *H,* H&E-stained sections. *A,* day 1 of involution; B, day 3; C, day 1; *D,* day 3; *E,* day 1; *F,* day 3; G, day 1; *H,* day 3. Note: the open alveolar structures present at day 1 involution are not present at day 3 involution. So/id *triangular arrows,* cells undergoing apoptosis. Cells undergoing apoptosis appear brown.

**Expression Patterns of the Apoptosis Pathway Genes bax and bcl-x during Involution Were Not Affected by the Absence of Functional p53.** Bax and bcl-x, induce apoptosis in cell culture (1 O-12). In related studies, we have determined that steady-state RNA levels of these genes increase coincident with apoptosis of mammary epithelial cells in *vivo* **(4, 6).** Total levels of bcl-xl+s increase associated with relative increases in the amount of bcl-x, expressed as compared to bcl-x,. During involution, the increase in expression of bcl-xl+S and bax was found during the initial stage of involution. After 10 days, involution is complete, and expression levels of these genes drop to levels comparable to those found during lactation (Fig. 5).

The absence of functional p53 did not affect the pattern of bax and *bcl-x* gene expression during involution (Fig. 5). At day 1 of involution, steady-state levels of bax and bcl-xL+s RNA were increased in Tag and p53 –/– mice, and by day 13, they had decreased (Fig. 5, *A* and B). At day 10 or 13 of involution, levels of bcl-xL+s were comparable to those found at day 1 of lactation. Relative levels of bcl-x, and bcl-x, RNA were evaluated using a RT-PCR analysis using primers that amplified both forms concurrently. The two forms were detected after hybridization using oligonucleotides specific for each form. This assay revealed that a relative increase in bcl-x, expression was found during involution in both the presence and absence of functional p53 (Fig. 5C). Although the relative levels of bcl-x, are increased,




Fig, 5. Patterns of bax and bcl-x gene expression during involution (I) in the presence and absence of functional p53 *A,* Northern blot analysis of steady-state levels of bax and bcl-xL +S RNA during involution in control and Tag mice. RNA from lactation day 1 (LI) is provided as a control. Ethidium bromide staining of 28S and 18s RNA is illustrated. B, Northern blot analysis of steady state levels of bax during involution in p53 +/+ and p53 −**I**− mice. Ethidium bromide staining of 185 RNA is illustrated. C, Southern blot analysis of bcl-x,- and bcl-x,-specific RT-PCR products during involution. Control and Tag mice are shown in the left panel. p53 +/+ and p53 **−/−** mice are shown in the *middle* and *right panels.* RNA from lactation day 1 (L1) is provided as a control.

bcl-x, was still the major form and was approximately 5- to IO-fold more abundant than bcl-xs

**The Absence of Functional p53 Did Not Affect Down-Regulation of Milk Protein Gene Expression during Involution.** With the onset of involution, the transcription of milk protein genes declines. To evaluate whether p53 participates in this aspect of involution, we measured the steady-state levels of milk protein RNAs during involution in the presence or absence of functional p53 There were no significant differences in the decline of milk protein RNAs in the presence or absence of functional p53 (Fig. 6).

## Discussion

**Roles for p53-independent and p53-dependent Apoptosis Pathways in the Mammary Gland.** During involution, the entire alveolar compartment of the mammary gland undergoes PCD, and the gland is remodeled to a virgin-like state. In this study, we have demonstrated that functional p53 is not required for this process. p53 mRNA has been detected in mouse mammary gland tissues (1). Evidence for both p53-independent and p53-dependent apoptosis pathways in mammary epithelial cells can be found in cultured mammary cell lines (7) and for p53-dependent apoptosis in transgenic mice that overexpress p53 in mammary tissue (6). However, the significance of these two pathways in normal mammary gland physiology and in disease had not been defined in vivo. By late pregnancy, the nearly empty fat pad of the virgin mouse is transformed into a tissue packed with lobulo-alveolar structures required for lactation. Following lactation, this structure is dismantled through apoptotic collapse, and the fat pad returns to a virgin state. The data presented here indicate that mammary gland involution does not require the presence of functional p53 This does not exclude the possibility that activation of p53-dependent pathways could be additive or play a recognizable role in different genetic backgrounds or under different developmental conditions (13).

With the demonstration that p53 is not required for mammary involution, it is necessary to focus attention on the protective role of p53 in proliferative disease of the breast. Absence of functional p53 is correlated with accelerated tumorigenesis (14, 15) and is associated with chromosome instability in the breast (14). Activation of p53-dependent apoptosis pathways may be one factor in determining the rate of growth of an individual breast tumor. The question is to what degree does activation of p53-independent apoptosis pathways affect tumor progression. In normal breast physiology, p53-independent pathways are readily activated. Does this mean they are also the first defense against an abnormal proliferative signal? Is p53 used to activate apoptosis only when this system fails? Or, do p53-independent and p53-dependent pathways operate concurrently? Since both p53-independent and p53-dependent pathways are associated with activation of some of the same downstream target genes (2-5, 16), it raises the possibility that simultaneous activation of both pathways increases the likelihood that cells will undergo apoptosis.

**The Patterns of bax and bcl-x Gene Expression during Involution Were Not Altered in the Absence of Functional p53** In tissue culture cells, p53 is a direct transcriptional enhancer of bax gene expression (5). In this and a related study (4), we have demonstrated that the steady-state levels of bax RNA are increased concurrently with apoptosis in the mammary gland, and that p53 is not required for the increase. Although not unexpected, this finding indicates that there are other important transcriptional activators of *bax* expression that mediate p53-independent transcription of the *bax* gene. There may be factors that influence steady-state levels of *bax* RNA in addition. Previous studies have defined a number of genes with increased expression levels during mammary gland involution (1, 4, 17-20)." From this selection of genes, only *bax* has been defined as a regulator of cell death in tissue culture cells (12). It is possible that some of the other genes known to be expressed during involution lie either upstream or downstream of bax expression. For example, overexpression of stromelysin-3 can trig-

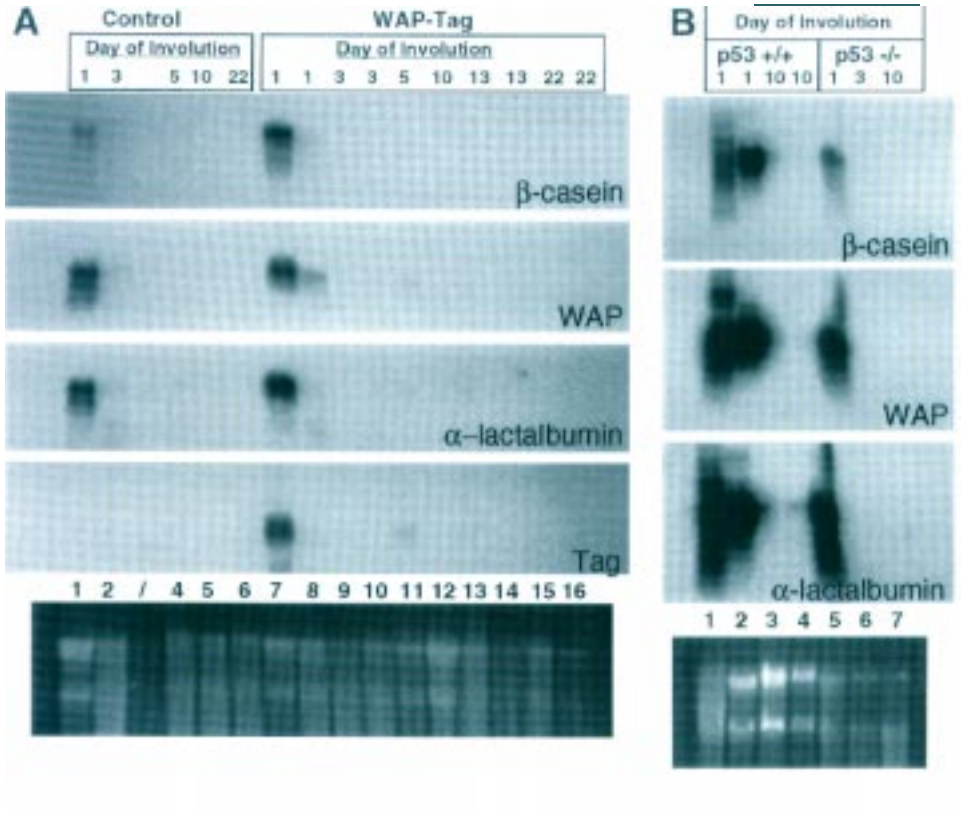



Fig. 6. Down-regulation of milk protein gene expression during involution in the presence and absence of functional p53 *A,* Northern blot analysis of steady-state levels of milk protein and Tag RNA during involution following the first pregnancy in nontransgenic control and WAP-Tag mice. B, Northern blot analysis of steady-state levels of milk protein RNA during involution in p.53 +I+ and p53 –/– mice. Milk proteins: b-casein, WAP, and a-lactalbumin. Ethidium bromide staining of 28s and 18s RNA is illustrated.

ger apoptosis in the mammary gland by disrupting extracellular basement membrane (19). It will be necessary to determine whether *bax* and stromelysin pathways are linked.

Bcl-x, is a survival factor in tissue culture cells and inhibits apoptosis (IO, 11). In cell culture, bcl-xs promotes cell death in a manner analogous to bax. However, its in *vivo* role is less clear because absolute expression levels of bcl-x, are far lower than bcl-x, (7, 8). In related studies, we have demonstrated increases in the relative amounts of bcl-x, RNA as compared to bcl-xL RNA, coincident with the onset of apoptosis in the mammary gland (5, 6). This suggests that apoptosis could be induced by a changing ratio of death inducer to death inhibitor molecules. In this study, we show that the change in ratio between the two bcl-x splice variants is unaltered in the absence of p53

In conclusion, mammary gland involution does not require functional p53 (Fig. 7). The remodeling process includes the disruption of basement membranes and extracellular matrix structures (21, 22), which is mediated by metalloproteinases, including stromelysin (19). The alveolar cells undergo p53-independent apoptosis. The genes encoding the death proteins bax and bcl-xs are activated with the onset of apoptosis in the involuting gland, rendering them candidates for the PCD pathway in mammary tissue. One elegant way of testing this hypothesis involves the temporally imbalanced expression of these genes in mammary tissue. We are currently attempting such an approach using the tetracycline-dependent temporal gene expression system (23, 24).

## Materials and Methods

**Mice, Induction of Involution, and Mammary Gland Biopsies.** Transgenic mice that carry a *WAP-Tag* hybrid gene and express Tag beginning at mid-pregnancy (Tag mice) have been established previously (9). The *WAP-Tag* transgene consists of a 1600-bp *WAP* gene promoter fragment linked to the SV40 early coding region containing the coding sequence for both large Tag and small Tag. Tag mice were studied after the first pregnancy. The mice that contained two defective germ-line p53 alleles contained a targeted disruption of the p53 gene (8).

Involution was induced in the Tag mice and nontransgenic control littermates by removing the pups directly after parturition. This time point was chosen because Tag mice are unable to lactate and, therefore, enter directly into involution after delivery. Involution was induced in the p53 -/- mice and breeding colony control p53 +/+ mice after 3 weeks of lactation by removing the pups.

Mammary gland biopsies were performed at 1, 3, and 13 days of involution in the Tag and control mice. Biopsies were performed in the p53 –/– and p53 +/+ mice at 1 and 3 days of involution. Mice were anesthetized using 0.7 ml of 0.175% avertin i.p. The inguinal mammary gland from either the right or left side was exposed and removed under sterile conditions. The mice recovered from the anesthesia uneventfully. Abdominal mammary glands were harvested at day 10 of involution in p53 - / - and p53 +/+ mice at the time of necropsy. These mice were killed by cervical dislocation.

**Mammary Gland Whole-Mount Preparations.** Each whole mammary gland specimen was spread on a glass slide and fixed in Carnoy's solution (100% ethanol:chloroform:glacial acetic acid, 6:3:1) for 60 min at room temperature. Following fixation, the glands were washed with 70% ethanol for 15 min, followed by a wash with distilled water for 5 min. The staining of the glands was performed in carmine alum (1 g carmine; Sigma Chemical Co., St. Louis, MO) and 2.5 g aluminum potassium sulfate (Sigma) in 500 ml water) at 4°C overnight. The tissues were then dehydrated and mounted on glass slides using routine methods.

**Histological Examination and Immunohistochemistry.** Mammary gland specimens were fixed in 10% neutral formalin solution and embed-

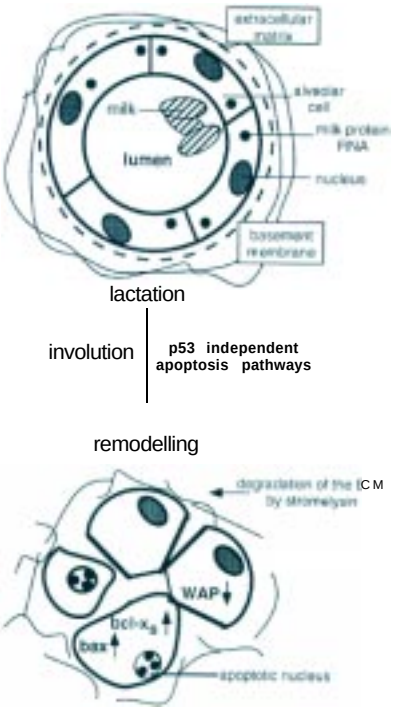



**Fig. 7.** Model Illustrating mammary gland involution and remodeling in the absence of functional p53 The mammary gland is prepared for lactation by the day of parturition. Alveolar cells are surrounded by basement membrane and extracellular matrix. Milk protein RNAs are transcibed, and milk is secreted into the alveolar lumen. During involution, the mammary gland is remodeled. p53-Independent apoptotic pathways can mediate this process. The extracellular matrix (ECM) is degraded by stromelysin. Cells exhibiting apoptotic nuclei appear. Expression of the apoptotic pathway genes bax (death inducer) and bc-xs (death inducer) increases. Expression of the milk protein genes (WAP, p-casein, and a-lactalbumIn) decreases. At the end of Involution, the gland is returned to a structure similar to that of a mature virgin.

ded in paraffin using routine methods. Five-pm tissue sections were prepared using routine methods for hematoxylin and eosin staining and for the detection of Tag protein. Tag protein was detected using the monoclonal antibody Pab 101 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA). Tissue sections were initially treated with pepsin (2-10 ug/ml in 0.01 N HCI buffer) for 15 min at room temperature and quenched with 0.03% H,O, in PBS for 30 min at room temperature. After treatment with normal horse serum for 30 min at room temperature, the specimens were incubated for 1 h with a 1 :I000 dilution of Pab101, followed by an incubation for 1 h with biotinylated horse antimouse IgG at a 1:400-500 dilution (Vectastain ABC kit; Vector, Laboratories, Inc., Burlingame, CA). The color reaction was performed with 0.05% 3,3'-dimethylaminoazobenzene (Sigma Chemical Co., St. Louis, Mo.) and 0.01% H,O,. Sections were counterstained with hematoxylin.

**Detection of Apoptotic Cell Death in Tissue Sections.** Mammary gland specimens were fixed and embedded; then 5-um tissue sections were prepared as described above. Apoptotic cell nuclei were identified using the Apotag kit (Oncor, Gaithersburg, MD). Sections were initially treated with 20 ug/ml proteinase K in PBS for 15 min at room temperature, quenched by 0.003% H,O, for 30 min at room temperature, equilibrated with buffer, incubated with TdT for 20-40 min at room temperature, washed with wash stop buffer for 30 min at 37"C, and incubated with anti-digoxigenin for 30 min at room temperature. Color was developed using 0.05% 3,3'-dimethylaminoazobenzene 0.01% H,O, diluted in 0.1 M Tris-HCI (pH 7.5) and counterstained with methyl green.

**Isolation of Total RNA, Northern Blot Analysis, and RT-PCR Assay.** Total RNA was isolated from individual mammary glands using acid-guanidinium thiocyanate-phenol-chloroform extraction (25), and the RNA was quantrtated on a spectrophotometer. For Northern blot analysis, 20 ug of each sample were fractionated on a formaldehyde agarose gel, transferred to a nylon membrane, and fixed on the membrane by UV irradiation. Gene expression was detected by hybridization of the membrane overnight with individual random primer-labeled probes. Gene expression was quantrtated using a radioanalytical Imaging system (AMBIS, Inc. San Diego, CA). The following 32P-labeled probes were used: WAP (26) b-casein (27), a-lactalbumin (28), bcI-xI+s (mRNA nt 1 10-394) and bax (mRNA nt 138-389). The relative amounts of bcl-x, and bcl-xs mRNA were measured using a RT-PCR assay. For the assays presented in Fig. 5, 1 ug of each sample was reverse transcribed, and the cDNA for bcl-xL+ was amplified using a pair of primers that amplify the nucleotide sequence containing the region differentrally spliced in the bcl-x, and bcl-x, mRNAs. For the assay presented, 25 cycles of PCR were used, and the signal obtained was proportional to the RNA input and number of PCR cycles. The 5' primer used corresponds to bcl-x mRNA nt 466-488 (5'-GCG CGG GAG GTG ATT CCC ATG GC-3') and the 3' primer used corresponds to bcl-x mRNA nt 891-870 (5'-CAT GCC CGT CAG GAA CCA GCG G-3'). The PCR products were fractionated on a 1.2% agarose gel, transferred to a nylon membrane, and fixed on the membrane by UV irradration. Expressron of bcl-x, was identified by hybridizing the membrane overnight with an oligonucleotide specific for the splice site contained within the 426-bp bcl-x, PCR product (5'-GGC GGG GCA CTG TGC GTG GAA AGC G-3'). Expression of bcl-xs was identified by hybridizing the membrane overnight with an oligonucleotide specific for the splice site contained within the 237-bp bcl-xs product (5'-CAG AGC TTT GAG CAG GAC ACT TTT GTG G-3'). Autoradiograph exposure times: bcl-x,, 5 h; bcl-xs, 5 h.

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